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Published in:
Applied Microbiology and Biotechnology

DOI:
10.1007/BF00176522

Publication date:
1990

Appl Microbiol Biotechnol (1990) 33:183–189

Applied and Microbiology Biotechnology

Biosynthesis of aromatic amino acids in *Nocardia* sp. 239: effects of amino acid analogues on growth and regulatory enzymes

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Received 23 August 1989/Accepted 19 December 1989

Summary. Further steps required for overproduction of aromatic amino acids by a mutant strain of *Nocardia* sp. 239 (Noc 87-13), unable to grow on L-phenylalanine as a sole carbon and energy source, were investigated. A number of analogues of the aromatic amino acids displayed severe inhibitory effects on the activities of regulatory enzymes in the biosynthetic pathway and growth of the organism in glucose mineral medium. L-Tryptophane analogues strongly inhibited 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase activity. L-Tyrosine analogues especially inhibited DAHP synthase and chorismate mutase, whereas L-phenylalanine analogues strongly inhibited chorismate mutase and prephenate dehydratase activity. Addition of the aromatic amino acids and their precursors chorismate, 4-hydroxyphenylpyruvate, phenylpyruvate and anthranilate, to the medium counteracted the growth inhibitory effect of specific analogues. The data indicate that ortho- (OFP) and para-fluoro-D,L-phenylalanine (PFP), and L-phenylalanine amide, are the most suitable analogues for the isolation of feedback-inhibition-insensitive prephenate dehydratase mutants. Attempts to isolate L-tyrosine and L-tryptophane auxotrophic mutants were only successful in the latter case, resulting in the selection of a stable anthranilate synthase-negative mutant (Noc 87-13-14). Uptake of aromatic amino acids in *Nocardia* sp. 239 most likely involves a common transport system. This necessitates the use of anthranilate, rather than L-tryptophane, as a supplement during the isolation of L-tyrosine auxotrophic and OFP- and/or PFP-resistant mutant derivative strains of Noc 87-13-14.

Introduction

Amino acid biosynthesis is generally controlled by feedback inhibition and/or repression mechanisms. Especially synthesis of aromatic amino acids is metabolically expensive and the pathways involved are tightly controlled (Garner and Herrmann 1983; Herrmann 1983; Shiio 1986). 3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, chorismate mutase and prephenate dehydratase are major control points in the synthesis of L-phenylalanine, although the pattern and degree of inhibition and/or repression varies to some extent amongst the various microorganisms studied. Overproduction of aromatic amino acids may be achieved following the deletion of these feedback regulatory mechanisms, via the isolation of auxotrophic strains and/or strains resistant to amino acid analogues. When following this approach, L-phenylalanine, L-tyrosine and L-tryptophane production levels of 19 g/l (Ozaki et al. 1985), 17.6 g/l (Hagino and Nakayama 1973) and 12 g/l (Hagino and Nakayama 1975), respectively, were reported with strains of *Corynebacterium glutamicum*.

Previously, we reported the isolation of mutants of *Nocardia* sp. 239 blocked in the L-phenylalanine catabolic pathway (de Boer et al. 1988). One of these mutants, strain Noc 87-13, a phenylalanine dehydrogenase- and phenylpyruvate decarboxylase-negative double mutant, is unable to grow on L-phenylalanine and phenylpyruvate as sole sources of carbon and energy. The kinetic properties of regulatory enzymes in the biosynthetic pathway for aromatic amino acids in *Nocardia* sp. 239 have been studied in detail (de Boer et al. 1989). The aim of the present study was to identify the most suitable further strategy for the isolation of mutants overproducing aromatic amino acids. For this purpose the inhibitory effects of amino acid analogues on the activities of regulatory enzymes and on growth of *Nocardia* sp. 239 were investigated. In addition, the isolation of L-tryptophane and L-tyrosine auxotrophic mutants was attempted.

Materials and methods

Microorganisms and cultivation. Nocardia sp. 239 wild-type, LMD 80.32, and derived mutant strains were used. The maintenance of these strains, the mineral medium used, the procedures followed

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for cultivation in batch cultures and preparation of cell-free extracts have been described previously (de Boer et al. 1988). The growth inhibitory effects of amino acid analogues were studied by conventional agar (1%, w/v) plating techniques, using glucose (10 m*M*) mineral agar plates (total volume 7.5 ml) inoculated with cells of a mid-exponential growth phase culture of wild-type *Nocardia* sp. 239 in liquid glucose (10 m*M*) mineral medium. The amino acid analogue concentrations used are indicated in the various experiments. Various compounds were added to the same agar plates to study their ability to counteract the growth inhibition exerted by the amino acid analogues (supplied at the minimal inhibitory concentrations). The following compounds were added, either separately or in mixtures, to the agar plates: Supplement 1, L-phenylalanine, L-tyrosine and L-tryptophane, 50 μg/ml of each; Supplement 2, anthranilate, phenylpyruvate, 4-hydroxyphenylpyruvate, 100 μg/ml of each; chorismate, 100 μg/ml; yeast extract, 1%, w/v. Growth was checked after 5 days of incubation at 37° C.

Enzyme assays. DAHP synthase (EC4.1.2.15), chorismate mutase (EC 5.4.99.5), prephenate dehydratase (EC 4.2.1.51) and anthranilate synthase (EC 4.1.3.27) were assayed as described previously (de Boer et al. 1989). The effect of analogues of aromatic amino acids on the activities of DAHP synthase, chorismate mutase and prephenate dehydratase was studied in extracts of cells grown in glucose (10 m*M*) mineral medium. The analogue to be tested was preincubated with the complete reaction mixture for 5 min before starting the reaction with phosphoenolpyruvate, chorismate and prephenate, respectively. The amino acid analogue concentrations used are indicated in the various experiments.

Isolation of auxotrophic mutants. The mutagenesis procedure with 1,2,7,8-diepoxyoctane described by de Boer et al. (1988) was used with the following modifications. Cells were pregrown on glucose, and agar plates containing 20 m*M* glucose and 0.25 μg/ml L-tryptophane or L-tyrosine were used. After incubation of the agar plates for 1–2 weeks, auxotrophic mutants will form white pinpoint colonies which can be clearly distinguished from the big yellow colonies of the wild-type. L-Tryptophane auxotrophic mutants were isolated from Noc 87-13. Mutants specifically blocked in anthranilate synthase activity were subsequently selected by screening for growth on glucose mineral agar containing 50 μg/ml anthranilate. Strains capable of growth when supplied with either L-tryptophane or anthranilate were checked for anthranilate synthase activity.

The same procedures were followed for the isolation of L-tyrosine auxotrophic derivative strains of Noc 87-13-14 (anthranilate synthase-negative mutant, see below). For this purpose, agar plates containing 20 m*M* glucose and 10 μg/ml anthranilate were supplemented with L-tyrosine to final concentrations of 0.25, 0.50 or 1.0 μg/ml.

Production of aromatic compounds. To investigate whether strains of *Nocardia* sp. 239 excreted aromatic compounds, cells were grown in mineral medium (25 ml) containing 20 m*M* glucose and 3 μg/ml anthranilate. The 100 ml erlenmeyer flasks were fitted with a stainless steel spring to prevent flocculation after depletion of anthranilate. Following anthranilate depletion the cultures were incubated for a further 24–48 h. Samples were taken at appropriate time intervals, filtered through a Millipore filter (0.2 μm pore size) and immediately placed on ice.

Analytical methods. Aromatic compounds excreted were analysed by HPLC using a μBondapack C18 or a μBondapack Phenyl column (de Boer et al. 1989). Protein concentrations in cell-free extracts were determined as described by Bradford (1976) with bovine serum albumin as a standard.

Biochemicals. Aromatic amino acids and their analogues, phenylpyruvate, 4-hydroxyphenylpyruvate, chorismate and anthranilate were obtained from Sigma Chemical Co. (St. Louis, Mo., USA) and 1,2,7,8-diepoxyoctane (97% solution) from Aldrich Chemical Co. (Brussels, Belgium).

Results

Inhibition of various enzymes by amino acid analogues in cell-free extracts

Previously (de Boer et al. 1989), we identified DAHP synthase, chorismate mutase and prephenate dehydratase as enzymes sensitive to feedback inhibition by aromatic amino acids in *Nocardia* sp. 239. We now observed that chorismate mutase was not only inhibited by L-phenylalanine (inhibition constant, $K_i = 60$ μ*M*) and L-tyrosine ($K_i = 35$ μ*M*), but also by a number of their analogues tested (Table 1). The enzyme was insensitive to L-tryptophane but inhibited by some of its analogues. Prephenate dehydratase was subject to inhibition by L-phenylalanine ($K_i = 10$ μ*M*) and most of its analogues, except *p*-chloro-D,L-phenylalaninol (PCP), L-phenylalaninol (PA), and *p*-amino-D,L-phenylalanine. The latter compound, plus L-tyrosine (activation constant, $K_a = 10$ μ*M*) and its related analogues, in fact stimulated activity of prephenate dehydratase. L-Tryptophane ($K_i = 600$ μ*M*) and 1-methyl-D,L-tryptophane inhibited prephenate dehydratase activity but related analogues had only a relatively minor effect, if at all. Only a single DAHP synthase enzyme is present in *Nocardia* sp. 239, sensitive to feedback inhibition by all three aromatic amino acids (de Boer et al. 1989). The enzyme was sensitive most of all to L-tryptophane ($K_i = 3$ μ*M*), L-tyrosine ($K_i = 180$ μ*M*), and their related analogues. The inhibitory effect of 1 m*M* L-phenylalanine ($K_i = 160$ μ*M*) was clearly stronger than that caused by its analogues.

Inhibition of growth by amino acid analogues

Most of the analogues of the aromatic amino acids tested were able to completely inhibit growth of wild-type *Nocardia* sp. 239 on glucose mineral agar plates (Table 2). To identify the metabolic processes affected, various compounds were added to the same agar plates to study their ability to compensate for (or to counteract) the growth inhibition exerted by the amino acid analogues supplied at their respective minimal inhibitory concentrations. Supplement mixtures of the amino acids L-phenylalanine, L-tyrosine and L-tryptophane, the biosynthetic intermediates anthranilate, phenylpyruvate, 4-hydroxyphenylpyruvate, and yeast extract were capable of reversing the inhibitory effect of a number of the analogues (Table 2). Each of these supplements completely reversed the inhibitory effects of the phenylalanine analogues ortho- (OFP) and para-fluoro-D,L-phenylalanine (PFP) and the tryptophane analogues α-methyl-D,L-tryptophane (MT) and D,L-7-azatryptophane (AT). Only addition of yeast extract allowed growth to occur on agar plates containing L-phenylalanine ethylester (PEE), PA and L-tyrosine ethylester.

Table 1. The effect of aromatic amino acids and their structural analogues on the activity of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase, chorismate mutase and prephenate dehydratase in extracts of glucose-grown cells of *Nocardia* sp. 239 wild-type

Amino acid (analogue)	Relative enzyme activity in presence of effector (1 m*M*)		
	DAHP synthase	Chorismate mutase	Prephenate dehydratase
None	1.00	1.00	1.00
L-Phenylalanine (L-Phe)	0.26	0.41	0.50
D-Phenylalanine	1.10	0.18	0.77
o-Fluoro-D,L-phenylalanine (OFP)	0.98	0.45	0.36
m-Fluoro-D,L-phenylalanine	0.97	0.43	0.21
p-Fluoro-D,L-phenylalanine (PFP)	0.93	0.65	0.21
p-Amino-D,L-phenylalanine	0.98	1.10	2.40
L-Phenylalanine amide (PAA)	1.00	0.25	0.27
β-2-Thienyl-D,L-alanine	0.92	0.84	0.59
L-Phenylalaninol (PA)	ND	0.93	0.93
L-Phenylalanine ethylester (PEE)	ND	0.53	0.38
p-Chloro-D,L-phenylalanine ethylester	ND	ND	0.54
p-Chloro-D,L-phenylalanine methylester	ND	ND	0.60
p-Chloro-D,L-phenylalaninol (PCP)	ND	ND	1.10
D,L-Phenylalanine hydroxamate	0.29	ND	ND
L-Tyrosine (L-Tyr)	0.40	0.50	3.70
D-Tyrosine	0.55	0.81	1.50
m-Fluoro-D,L-tyrosine	0.58	0.66	2.40
3-Chloro-L-tyrosine	ND	1.20	2.40
L-Tyrosine ethylester (TEE)	ND	1.00	3.10
L-Tyrosinol	ND	0.94	1.40
L-Tyrosine hydroxamate	ND	0.69	1.80
L-Tryptophane (L-Trp)	0.06	1.00	0.50
D-Tryptophane	0.08	0.88	0.91
α-Methyl-D,L-tryptophane (MT)	ND	0.79	0.89
1-Methyl-D,L-tryptophane	0.43	0.85	0.40
5-Methyl-D,L-tryptophane	0.20	0.99	1.10
6-Methyl-D,L-tryptophane	0.24	0.80	1.10
7-Methyl-D,L-tryptophane	0.51	0.71	0.73
D,L-7-Azatryptophane (AT)	0.61	0.97	1.90
6-Fluoro-D,L-tryptophane	0.41	0.86	0.93
D,L-Tryptophane hydroxamate	0.04	0.80	ND

ND = not determined

To identify the compounds in the supplement mixtures that reversed growth inhibition, the various components were added separately or in combinations to the glucose agar plates containing the amino acid analogues (Table 3). Growth inhibition by OFP was readily reversed by not only L-phenylalanine but also by chorismate and phenylpyruvate. The same pattern was observed in PFP-containing agar plates, but in this case the stimulating effect of L-phenylalanine was less clear. Inhibition of growth by MT and AT was totally overcome by the presence of L-tryptophane and its precursor anthranilate. Interestingly, the inhibitory effects of PCP and L-phenylalanine amide (PAA) were only compensated by addition of chorismate and chorismate plus phenylpyruvate, respectively (Table 3).

Isolation of L-tryptophane auxotrophic mutants

L-Tryptophane auxotrophic mutants were derived from Noc 87-13 following treatment with 1,2,7,8-diepoxyoctane as the mutagen. When applying the rapid screening method described in Materials and methods, 11 mutants affected in L-tryptophane biosynthesis were isolated among 260,000 colonies tested. The characterization of these mutants showed that strains Noc 87-13-11, Noc 87-13-12 and Noc 87-13-14 totally lacked anthranilate synthase activity. Growth of these mutants on glucose mineral agar plates only occurred after the addition of anthranilate or L-tryptophane. The other mutants clearly possessed significant anthranilate synthase activities and could only be supplemented by addition of L-tryptophane (Table 4). The most stable anthranilate synthase-negative mutant (strain Noc 87-13-14) was selected for further studies. No overproduction of L-phenylalanine, or other aromatic compounds, could be detected during growth of the latter mutant in glucose mineral medium supplemented with anthranilate (or L-tryptophane).

Table 2. The effect of medium supplementation on the growth of wild-type *Nocardia* sp. 239 on glucose (20 m*M*) mineral agar plates containing aromatic amino acid analogues at minimal inhibitory concentrations

Amino acid analogue	Minimal inhibitory concentration (mg/ml)	Growth supplement			
		None	Sup. 1[a]	Sup. 2[b]	YE[c]
o-Fluoro-D,L-phenylalanine	5.0	–	+	+	+
m-Fluoro-D,L-phenylalanine	5.0	–	–	–	–
p-Fluoro-D,L-phenylalanine	4.0	–	+	+	+
D,L-Phenylalanine hydroxamate	0.5	–	–	–	–
β-2-Thienyl-D,L-alanine	5.0	–	–	–	–
L-Phenylalanine amide	7.0	–	–	+	+
p-Chloro-D,L-phenylalanine methylester	2.0	–	–	–	–
p-Chloro-D,L-phenylalanine ethylester	1.5	–	–	–	–
p-Chloro-D,L-phenylalaninol	0.3	–	–	–	+
L-Phenylalanine ethylester	3.0	–	–	–	+
L-Phenylalaninol	1.0	–	–	±	+
L-Tyrosine ethylester	2.0	–	–	–	+
L-Tyrosinol	5.0	–	–	–	–
5-Methyl-D,L-tryptophane	2.0	–	–	–	–
α-Methyl-D,L-tryptophane	1.0	–	+	+	+
D,L-7-Azatryptophane	1.5	–	+	+	+
6-Fluoro-D,L-tryptophane	4.0	–	–	–	–
D,L-Tryptophane hydroxamate	0.5	–	–	–	–
L-Tryptophane amide	0.5	–	–	–	–
L-Tryptophane ethylester	1.0	–	–	–	–
L-Tryptophane methylester	2.5	–	–	–	–

No inhibition of growth was observed with D-phenylalanine, *p*-amino-D,L-phenylalanine, *p*-nitro-L-phenylalanine, L-phenylalanine methylester, D-tyrosine, *m*-fluoro-D,L-tyrosine, 3-chloro-L-tyrosine, D-tryptophane, and 1-, 6-, and 7-methyl-D,L-tryptophane

[a] Sup. 1 = Supplement 1 (50 μg/ml each of L-phenylalanine, L-tyrosine, and L-tryptophane)

[b] Sup. 2 = Supplement 2 (100 μg/ml each of anthranilate, phenylpyruvate, and 4-hydroxyphenylpyruvate)

[c] YE: yeast extract (1% w/v)

Growth of Noc 87-13-14 in the presence of aromatic amino acids

The further isolation of L-tyrosine auxotrophic derivative mutants of Noc 87-13-14 resistant to aromatic amino acid analogues appears to be required in order to achieve overproduction of L-phenylalanine. For this purpose, strains will have to be grown routinely in the presence of L-tryptophane, or anthranilate, and L-tyrosine as auxotrophic supplements, plus various analogues of L-phenylalanine under conditions which may result in the accumulation of L-phenylalanine. The availability of anthranilate synthase-negative mutants allowed us to investigate whether L-tryptophane or anthranilate uptake was inhibited by the presence of L-phenylalanine and L-tyrosine. Growth of Noc 87-13-12 and Noc 87-13-14 on glucose mineral agar plates supplemented with L-tryptophane (0.25 μg/ml) was completely blocked in the presence of a 400-fold excess of L-phenylalanine and/or L-tyrosine (100 μg/ml). Normal growth, however, occurred when anthranilate (0.25 μg/ml) was used as the supplement. These observations clearly suggest that L-tryptophane uptake is inhibited by the other aromatic amino acids. Most likely a common transport system is involved in the uptake of these amino acids. It was therefore decided to use anthranilate rather than L-tryptophane as the supplement in further experiments attempting to isolate L-tyrosine auxotrophic and analogue-resistant mutants of Noc 87-13-14.

Isolation of L-tyrosine auxotrophic mutants

Following treatment of cells of Noc 87-13-14 with 1,2,7,8-diepoxyoctane, appropriate dilutions were plated out on glucose mineral agar plates containing anthranilate (10 μg/ml) plus L-tyrosine (0.25, 0.50 or 1.0 μg/ml). After 1–2 weeks of incubation the plates were checked for the presence of white pin-point colonies, as outlined above. At each L-tyrosine concentration about 500,000 colonies were screened, but no tyrosine auxotrophs could be detected.

Discussion

The development of fermentative processes for amino acid production is often greatly facilitated by the isolation of bacterial strains resistant to amino acid ana-

Table 3. Growth of wild-type *Nocardia* sp. 239 on glucose (20 m*M*) mineral agar plates containing aromatic amino acid analogues and supplements

Supplement	Growth in the presence of[a]:							
	OFP	PFP	MT	AT	PAA	PCP	PEE	PA
L-Phe[b]	+	±	−	−	−	−	−	−
L-Tyr[b]	−	−	−	−	−	−	−	−
L-Trp[b]	−	−	+	+	−	−	−	−
L-Phe + L-Tyr	+	+	−	−	−	−	−	−
L-Phe + L-Trp	+	+	+	+	−	−	−	−
L-Tyr + L-Trp	−	−	+	+	−	−	−	−
Supplement 1[c]	+	+	+	+	−	−	−	−
Chorismate[d]	+	+	−	−	+	+	−	−
Anthranilate[d]	−	−	+	+	−	−	−	−
Phenylpyruvate[d]	+	+	−	−	+	−	−	−
4-Hydroxyphenylpyruvate[d]	−	−	−	−	−	−	−	−
Supplement 2[c]	+	+	+	+	+	−	−	−
Yeast extract[e]	+	+	+	+	+	+	+	+

For all abbreviations see Table 1

[a] Present at minimal inhibitory concentrations, see Table 2
[b] Final concentrations 50 μg/ml each
[c] Supplements 1 and 2 composition as indicated in Table 2
[d] Final concentrations 100 μg/ml
[e] Final concentration 10 mg/ml

logues. These analogues, however, may interfere with various cellular processes. The most obvious targets for inhibition are (the activity and/or synthesis of) amino acid transport systems, amino acid biosynthetic enzymes, and enzymes of the protein-synthesizing machinery (e.g. aminoacyl-tRNA synthase). Alternatively, analogues may become incorporated into proteins, conceivably resulting in changes in protein structure and loss of enzyme function.

The isolation of analogue-resistant mutants is a time-consuming process and, not surprisingly in view of the above, may meet with varying degrees of success with respect to amino acid overproduction. The present study was carried out with the aim of identifying analogues that specifically affect the activities of key regulatory enzymes (DAHP synthase, chorismate mutase and prephenate dehydratase) in aromatic amino acid biosynthesis in *Nocardia* sp. 239 and exert a clear

Table 4. Growth of wild-type *Nocardia* sp. 239, strain Noc 87-13 (L-phenylalanine dehydrogenase- and phenylpyruvate decarboxylase-negative), and derived L-tryptophane auxotrophic mutants, on glucose (20 m*M*) mineral agar plates supplemented with anthranilate or L-tryptophane, and anthranilate synthase activities in extracts of cells grown on glucose (20 m*M*) mineral medium supplemented with 25 μg/ml L-tryptophane

Strain	Growth after supplementation[a] with:			Anthranilate synthase activity[b]
	None	Anthranilate	L-Tryptophane	
Wild-type	+	+	+	1.1
Noc 87-13	+	+	+	0.9
Noc 87-13- 4	−	−	+	1.3
Noc 87-13- 7	−	−	+	1.0
Noc 87-13- 8	−	−	+	0.7
Noc 87-13- 9	−	−	+	1.2
Noc 87-13-10	−	−	+	0.9
Noc 87-13-11	−	+	+	ND[c]
Noc 87-13-12	−	+	+	ND
Noc 87-13-14	−	+	+	ND
Noc 87-13-15	−	−	+	0.8

[a] Anthranilate and L-tryptophane were supplemented to give final concentrations of 50 μg/ml
[b] Enzyme activities expressed in nmol min^{-1} mg^{-1} protein
[c] ND = activity not detectable

growth inhibitory effect. No evidence is available at the moment to suggest that these enzymes are additionally regulated at the level of their synthesis. A number of structural analogues of the aromatic amino acids were found to inhibit both the activities of these enzymes and growth of *Nocardia* sp. 239 (Tables 1 and 2). The growth inhibitory effects of the phenylalanine analogues OFP and PFP were counteracted by phenylpyruvate and L-phenylalanine (Table 3). Both OFP and PFP were found to strongly inhibit prephenate dehydratase activity (Table 1) and it is therefore concluded that these analogues block L-phenylalanine biosynthesis, and thus growth, at the level of prephenate dehydratase.

The stimulating effect of chorismate was rather puzzling at first. HPLC analysis, however, showed that the commercial preparation of this compound contained significant amounts of phenylpyruvate. The growth inhibitory effects of the tryptophane analogues MT and AT were completely reversed by the addition of anthranilate or L-tryptophane. The most likely target for these analogues is thus anthranilate synthase, preventing L-tryptophane synthesis, which will result in growth inhibition.

Inhibition by PCP could only be reversed by addition of chorismate, which suggests that this compound inhibits enzymes in the common (shikimate) pathway of aromatic amino acid biosynthesis. A possible effect of PCP on DAHP synthase could not be tested since it already interfered with the assay for this enzyme. The failure of aromatic amino acids and their precursors to counteract PCP inhibition indicates that other metabolites essential for growth (e.g. vitamins) are produced from chorismate.

PAA is a strong inhibitor of chorismate mutase and prephenate dehydratase and only the presence of phenylpyruvate reversed its growth inhibition. Most likely this compound additionally inhibits phenylalanine uptake. Among the many analogues tested, only the growth inhibitory effects of OFP, PFP, MT, AT, PCP and PAA could be compensated by the addition of single compounds. The results of the present study indicate that the aromatic amino acids, their precursors, and the analogues OFP, PFP, MT, AT, PCP and PAA readily enter cells of *Nocardia* sp. 239.

Several analogues were found to inhibit enzyme activities, but their growth inhibitory effect was not reversed by the addition of yeast extract or any other supplement tested (see Table 2; e.g. *m*-fluoro-D,L-phenylalanine, D,L-phenylalanine hydroxamate and D,L-tryptophane hydroxymate). Conceivably, these analogues also interfere with other essential steps in cellular metabolism referred to above (e.g. the protein synthesizing machinery).

The availability of mutants of *Nocardia* sp. 239 blocked in the aromatic amino acid biosynthetic pathway will greatly facilitate further work aiming to clone the genes involved. As mentioned above, L-tryptophane is a strong inhibitor of DAHP synthase. The introduction of a block in anthranilate synthase will not only allow a further channelling of carbon flow towards the L-phenylalanine and L-tyrosine specific branch of the pathway, but also serve to avoid any feedback inhibitory effects of L-tryptophane on DAHP synthase activity. The isolation of L-tryptophane auxotrophic mutants from Noc 87-13 was relatively easy (Table 4), but subsequent attempts to isolate L-tyrosine auxotrophic derivative mutants met with failure. Evidence is available in the literature indicating the presence of transport systems possessing affinity for all three aromatic amino acids in various bacteria (Ames and Roth 1968; Brown 1970; Kay and Gronlund 1971). Addition of L-tyrosine or L-phenylalanine to growth media of Noc 87-13-14 containing L-tryptophane as the auxotrophic supplement blocked growth completely. However, growth proceeded normally when L-tryptophane was replaced by anthranilate. These observations suggest that a common transport system for aromatic amino acids is present also in *Nocardia* sp. 239. It was therefore decided to use anthranilate rather than L-tryptophane for the isolation of L-tyrosine auxotrophic mutants. Despite several attempts, using various supplement concentrations and screening a total of 1,500,000 colonies, no L-tyrosine auxotroph was isolated. A possible explanation is that L-tyrosine biosynthesis in *Nocardia* sp. 239 proceeds via dual routes, namely via 4-hydroxyphenylpyruvate and pretyrosine (arogenate) as intermediates. Dual pathways of L-tyrosine (and L-phenylalanine) biosynthesis have been found, for instance in *Pseudomonas aeruginosa* (Patel et al. 1977), making the isolation of auxotrophic mutants problematic (Patel et al. 1978; Berry et al. 1987). Alternatively, the aromatic amino acid aminotransferase involved in tyrosine biosynthesis may fulfil an essential role in other pathways as well (e.g. in phenylalanine biosynthesis).

Acknowledgements. The investigations were supported in part by the Foundation for Fundamental Research (BION) which is subsidized by the Netherlands Scientific Organization (NWO). Thanks are due to Professor W. Harder for valuable discussions.

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